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PRINCIPAL INVESTIGATOR: Michael Reiss, M.D.

CONTRACTING ORGANIZATION: Yale University
New Haven, Connecticut 06520-8047

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Michael Reiss, M.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Yale University
New Haven, Connecticut 06520-8047

E-MAIL:
reissmi@umdnj.edu

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Transforming Growth Factor-ß (TGFß) is the most potent known inhibitor of cell cycle progression of normal mammary epithelial cells. In general, advanced breast cancers are refractory to TGFß-mediated growth inhibition. The TGFß type I and type II receptors (TßR-I and -II) are the primary transducers of TGFß's antiproliferative effects. It is our working hypothesis that TGFß-resistance is caused by lesions in the TßR genes.
Using touch preps of primary breast carcinomas, we showed that approximately 50% of primary breast carcinomas contain subpopulations of cells that have lost one or both copies of the TßR-I or -II gene. Screening of the TßR-I and -II genes for the presence of mutations using PCR-SSCP and DNA sequencing indicate that a particular missense mutation (S387Y) in TßR-I is present in approximately 6% of primary cancers and 42% of axillary lymph node metastases. Finally, we have generated antibodies that specifically recognize the activated (phosphorylated) forms of Smad2 and -3. These allow us to assess in situ whether breast cancer cells are responding to TGFß, and whether TßR defects are present or not. Approximately 12% of primary breast cancers fail to activate Smad2, indicating the presence of a TGFß receptor defect.

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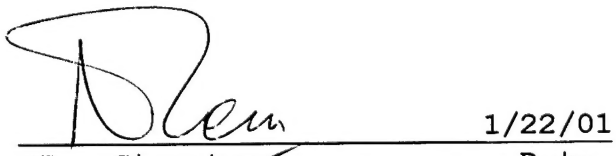

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INTRODUCTION

Transforming Growth Factor-ß (TGFß) is the most potent known inhibitor of cell cycle progression of normal mammary epithelial cells; in addition, it causes cells to deposit increased amounts of extracellular matrix, which affects cell-cell and cell-substrate interactions. In general, advanced breast cancers are refractory to TGFß-mediated growth inhibition, while the TGFß they secrete apparently serves to enhance invasion into surrounding structures and perhaps their metastatic potential. The effects of TGFß on cell cycle progression are transduced by two cell surface receptors, TGFß type I (TßR-I) and -II receptors (TßR-II), and relayed from the membrane to the cell nucleus by three recently discovered members of the MAD family of proteins, Smad2, -3, and -4. It is our working hypothesis that TGFß-resistance can, in principle, be caused by molecular lesions in any of these five genes, that such lesions are likely to occur during the development or progression of human breast cancer, and that they may impact on prognosis or treatment response.

This project addresses three of the fundamental research issues raised by the USAMRMC Breast Cancer Research Program. The first question is whether or not molecular lesions of the genes involved in the TGFß signaling pathway contribute to the origin and/or progression of breast cancer. We expected changes in these genes to be relatively late events, perhaps characteristic of metastatic cancer. Secondly, we proposed to determine how molecular lesions in the TGFß receptor and/or Smad genes affect receptor function, and how they might play a role in the development and/or progression of breast cancer. Thirdly, we intended to examine the question whether genetic lesions in TGFß receptor and/or Smad genes are able to predict the outcome of patients with breast cancer. Because the anti-tumor effects of anti-estrogens such as tamoxifen are thought to be mediated by the auto- and paracrine induction of TGFß, we wished to test the hypothesis that resistance of hormone-receptor positive cancers to tamoxifen is the result of inactivation of TGFß pathway genes.

BODY

The Statement of Work in our original proposal included the following tasks/timeline:

Task 1. Screening for mutations in TGFß receptor genes in breast cancer

a. Identification of genetic alterations of TGFß-receptor genes in invasive breast cancer specimens. - Months 1-24

b. Identification of genetic alterations of TGFß-receptor gene in sets of pre-invasive, primary invasive and metastatic (lymph node positive) breast cancers in order to determine the stage of tumor development at which these mutations occur. Months 12-36.

Task 2. Determination of the functional consequences of TGFß-receptor mutations

Cloning of TGFß-receptor mutants into mammalian expression vectors and transfection into TGFß-sensitive and -resistant human mammary epithelial cells to determine whether the mutations are dominant or recessive, and correlation of the site of mutations within the molecule with the way they affect the cellular phenotype. - Months 12-36

Task 3. To determine the potential clinical significance of genetic alterations of the TßR genes in breast cancer

Test the hypothesis that genetic alterations of TGFß-receptor genes predict for resistance to anti-estrogen therapy in patients with estrogen-receptor positive tumors. Months 36-48.

Progress achieved on each of these tasks will be described separately:

Task 1. Screening for mutations in TGFß receptor genes in breast cancer

Our initial studies of genes involved in TGFß signaling focused on the TßR-II gene. Using a chemical mismatch cleavage (CCM) assay, we were the first to identify missense mutations within the TßR-II serine-threonine kinase domain in human cancer cell lines {Garrigue-Antar, 1995 #717}. These findings raised two important questions: (1) Do such structural alterations of the TßR genes also occur in primary tumors (particularly

breast cancers) *in vivo*? and, if so (2) How do mutations in the TßR genes affect receptor function?

a. GENOMIC ANALYSIS

Selection of breast cancers for genomic analysis. In collaboration with our breast pathologist, Dr. Darryl Carter, we selected a series of 36 primary stage I and -II breast carcinoma specimens for which both frozen and paraffin-embedded material is available. In 12 of these cases, we also had lymph node metastatic lesions available for analysis. We have completed the molecular structural analysis of the TßR-I and -II genes in this series. The final results are presented here:

Tissue specimens and nucleic acid extraction: Breast carcinoma specimens were provided by the Program for Critical Technologies in Breast Oncology at Yale after hisopathological review by one of us (D.C.). Genomic DNA was extracted from tumor and normal tissues as previously described (18). Isolating genomic DNA from a single 5 µm microdissected paraffin-embedded tumor section using InstaGene matrix (Bio-Rad, Hercules, CA) typically yielded 200 µl of DNA template solution. Total cellular RNA was extracted from three or more 50 µm serial thick frozen sections using TriZOL® reagent (GIBCO-BRL).

Genotyping of TGFß signaling intermediates: The TßR-II gene was analyzed by chemical mismatch cleavage as previously described (19), or by conventional PCR-SSCP (For primers used to amplify TßR-II exons. The TßR-I gene was analyzed by "cold" PCR-SSCP. In this case, each 20-µl PCR reaction contained 500 nM of unlabeled primers. Following an initial 3 minute denaturation at 95°C, PCR was performed for 35 cycles of 95°C for 30 seconds, 55°C for 40 seconds, 72° for 30 seconds followed by a 5 min final extension at 72°C. For PCR amplification of the GC-rich exon 1 we used the Advantage-GC genomic polymerase mix (Clontech Palo Alto, CA) according to the instructions supplied by the manufacturer. The 9 exons of the TßR-I gene were amplified using the following flanking intronic forward and reverse primers: Exon 1: 5'-gaggcgaggtttgctggggtgaggca-3' and 5'-catgtttgagaaagagcaggagcgag-3'; exon2: 5'-ctacacaatctttctctttttcc-3' and 5'-gtttttcttgtagtatctagg-3'; exon 3: 5'-gtttatttcactcgaggcc-3' and 5'-ggagaaacaattatgttac-3'; exon 4: 5'-gattgtgttgagtactattta-3' and 5'-ggaaaagcaaatgttacagac-3'; exon 5: 5'-gcccaaccgaaatgttaattc-3' and 5'-ggtagaactgcttatagaat-3'; exon 6: 5'-gcagtcatgtttaatttttgattc-3' and 5'-gaacgcgtattaaatatagttg-3'; exon 7: 5'-tgtctgaaaggaggttcatcc-3' and 5'-gaacaacttctgctcatgacg-3'; exon 8: 5'-gccttgcattagctgaataaat-3' and 5'-gcttactaagcagaagcag-3'; exon 9: 5'-ggaaaatggtgcatgcatta-3' and 5'-gagttcaggcaaagctgtag-3'. For SSCP analysis, 5 µl aliquots of amplified PCR product were mixed with 15 µl loading buffer (12.5 µl 10x TBE buffer, 2 µl of 15% Ficoll, 0.1% bromophenol blue & xylene cyanol, 0.5 µl methyl mercury hydroxide), denatured by heating at 80°C for 3 minutes, and quenched on ice. The single stranded DNA fragments were then resolved using precast 20% TBE acrylamide gels on a Novex Xcell II Thermoflow apparatus (Novex, San Diego, CA) with the gel temperature precisely maintained at 10°C throughout the run. Bands were visualized by staining the gel in a 1:10,000 dilution of SYBR™ Green II (Molecular Probes, Inc., Eugene, OR) for 20-30 minutes and using an Eagle Eye charged coupled device camera equipped with a SYBR™ Green band pass filter (Stratagene) for photographic documentation.

Suspect bands were excised from the gels with a razor blade and reamplified. PCR products were purified using the QIAquick PCR purification kit (QIAGEN, Chatsworth, CA), and subjected to DNA sequencing using a thermocycling sequencing kit (Epicentre® Technologies, Madison, WI) with either a forward or reverse primer end-labelled with [γ-^{32}P]-ATP. Reaction products were denatured at 70°C for 3 minutes, resolved on 7% (w/v) denaturing polyacrylamide gels at 50°C and visualized by exposing dried gels to X-ray film overnight at 20°C. The presence of any sequence alteration was always confirmed by repeated PCR-SSCP and DNA sequencing using an independent aliquot of tumor-derived genomic DNA as template. Whether any mutations were somatic in nature or present in the germline was determined by analyzing genomic DNA isolated from non-cancer tissue of the same patient.

TßR Gene Expression in Primary Breast Cancer: In order to test the hypothesis that breast carcinomas *in vivo* are refractory to TGFß, we analyzed the molecular characteristics of the two cell surface receptor genes, TßR-I and -II. TßR expression was determined using a reverse transcription-PCR assay in 14 frozen surgical breast cancer specimens from which we were able to extract good quality RNA. Each of these samples expressed both TßR-I and -II mRNA transcripts (data not shown). This is in contrast to our previous studies in esophageal- and small cell lung cancers, in which loss of TßR-II mRNA was found in 25% and 100% of cases, respectively.

Structural Analysis of the TßR-II Gene in Primary Breast Cancer: The entire open reading frame of TßR-II was screened for the presence of mutations by chemical mismatch cleavage or by PCR-SSCP followed by

DNA sequencing. No DNA sequence alterations were encountered in a total of 30 cases examined. Thus, the TßR-II gene is normally expressed in primary human breast cancer, and mutations of this gene are probably rare. This result is perhaps not surprising in light of previous studies of other cancer types: Missense and/or nonsense mutations in the TßR-II gene have only been found sporadically in colorectal- and head-&-neck cancers and in cutaneous T-cell lymphomas. The only exceptions are tumors that are associated with DNA mismatch repair deficiencies which frequently display TßR-II nonsense mutations.

Structural Analysis of the TßR-I Gene in Primary Breast Cancer: In order to determine whether mutations in the TßR-I gene might be found in human breast cancer, we screened each of the 9 exons of the TßR-I gene by PCR-SSCP in 31 primary breast carcinoma specimens and in 12 associated lymph node metastases. Areas of tumor tissue were isolated from paraffin sections by microdissection and the remaining surrounding breast tissue was used to extract germline genomic DNA. Individual exons were amplified by PCR and the products screened for the presence of novel single-strand conformation polymorphisms. Suspect bands were re-amplified and subjected to direct DNA sequencing. Individual sequence abnormalities were confirmed by repeating the entire procedure using a second aliquot of genomic DNA.

Our most important finding was a C to A transversion at nucleotide 1160 in exon 7 of TßR-I, which predicts for a serine to tyrosine substitution at codon 387. This was the only mutation encountered in the entire series and was present in 7 of the 43 specimens (16%, 95% CI: 7-31%). This is the first report of a mutation in the TßR-I gene in any type of human malignancy. Moreover, this mutation may be specifically associated with breast cancer as we have not found it in any cervical carcinomas nor in head-&-neck cancer cell lines (V.F. Vellucci and M. Reiss, unpublished data). In addition, Pasche et al. recently reported the absence of TßR-I mutations in acute myeloid leukemias.

Our second major finding is the highly significant association between the S387Y mutant and axillary lymph node metastases: While we encountered this mutation in only 2 of 31 (6%, 95% CI: 1-21) primary breast cancer specimens, it was present in 5 of the 12 (41%, 95% CI: 15-72) lymph node metastases (Fisher's Exact Test, p=0.012). The dramatically increased frequency of this mutation in lymph node metastases indicates that inactivation of the TGFß pathway may represent a late event in breast cancer progression. The fact that most breast carcinoma cell lines are refractory to TGFß is also consistent with this idea, as most of these cell lines were initially derived from metastatic cells isolated from malignant pleural effusions or ascites. Moreover, in animal models of skin carcinogenesis, TGFß resistant tumor cell clones also do not emerge until the tumors have become highly aggressive and metastatic. In contrast, in colorectal cancers associated with DNA mismatch repair deficiencies, the acquisition of TßR-II gene mutations appears to coincide with the transition from pre-invasive adenoma to invasive carcinomas. Thus, the stage of tumor development at which the TGFß signaling pathway becomes inactivated appears may vary depending on the tumor type and on the underlying molecular genetic events that drive carcinogenesis.

As the S387Y mutation was not detected in germline DNA of the same individuals, we can practically exclude the possibility that this sequence alteration represents a normal polymorphism. On the other hand, besides the mutant band, a wild type band could be detected in each of the tumor specimens. Although these findings suggest that the tumors may have retained a wild type allele, it is impossible to exclude the possibility that this wild type band was the result of the almost inevitable contamination of the specimens with at least some normal cells. However, even loss of function of one of the two TßR-I alleles may be sufficient to confer a significant a selective advantage. Such a dosage effect occurs, for example, in transgenic animals that express a dominant-negative TßR-II gene in conjunction with two endogenous wild type alleles, and in knock-out mice that carry only a single TGFß1 gene allele. In both of these situations, the animals are significantly more susceptible to tumor formation.

Besides the S387Y somatic missense mutation, we also detected a variant allele of the TßR-I gene with an in-frame deletion of 3 of 9 repeating GGC trinucleotides within exon 1. Thirteen of 24 evaluable cases with BC were heterozygous carriers of this del$(GGC)_3$ TßR-I variant (54%, 95% C.I. 33-74%). This deletion results in the loss of 3 of the 9 alanine residues that constitute the hydrophobic core of the putative TßR-I signal. Comparative hydrophobicity plots of wild type and the del $(GGC)_3$ TßR-I variant clearly show that the deletion shortens the hydrophobic core of the signal peptide. These findings suggested that this deletion might well have functional consequences for the receptor protein, particularly its ability to be targeted to the cell membrane.

In order to determine whether there might be an association between the carrier state of the del$(GGC)_3$

TßR-I variant and the development of breast cancer, we determined the frequency of the del(GGC)$_3$ allele in a cohort of germline DNA samples from 43 independently and randomly selected individuals. Only one of these individuals was heterozygous for the del(GGC)$_3$ variant of TßR-I (2%, 95% CI: 0-12%). This translates into a highly significant increased relative risk of developing BC in carriers over control (Fisher's Exact test: $p<0.0001$)(Relative risk: 3.18; 95% C.I.: 2.32-4.36). These findings strongly argue in favor of the hypothesis that the del(GGC)$_3$ variant of TßR-I confers an increased cancer risk, presumably by decreasing the sensitivity of normal breast epithelial cells to TGFß.

Case-control Study: In order to test the validity of these results, we have taken advantage of the recently completed Yale Environment and Breast Disease Study. In this prospective case-control study, Dr. Tongzhang Zheng has been testing the hypothesis that exposure to organochloride pesticides increases the risk of BC. Close to 400 cases and 200 control women were enrolled between January 1994 and August 1997. All cases had histologically confirmed diagnoses of primary BC (TNM stages 0-III). Standardized structured questionnaires were used to ascertain demographic factors, menstrual and reproductive history, past medical history and family history of cancer, occupation, household pesticide use, use of hair dyes, alcohol and tobacco, and dietary history. In addition, blood clots were stored frozen to be used for future studies of genetic polymorphisms. The epidemiological data that have been collected and the availability of genomic DNA from all cases and controls represented an invaluable opportunity for us to rigorously test the idea that the del(GGC)$_3$ TßR-I gene variant may represent a novel and common breast cancer susceptibility gene.

Cases (n=98) were selected from among previously ascertained subjects who participated in the Yale Environment and Breast Disease Study. All cases had histologically confirmed primary BC (stages 0-III). Age-matched controls (n=92) were selected from among the women in the same study who did not have a diagnosis of BC. Eleven cases (11%, 95% CI: 6-19%) and 14 of the controls (15%, 95% CI: 9-24%) were heterozygous carriers of the del(GGC)$_3$ TßR-I gene variant. These results indicate that there was no significant association between the del(GGC)$_3$ TßR-I gene variant carrier state and breast cancer (Fisher's Exact test, $p=0.52$).

In summary, in this initial series of primary breast cancers, we identified one particular structural alterations of the TßR-I gene that appears to be uniquely associated with breast carcinomas, and is found more frequently in axillary lymph node metastases than in primary tumors. In order to confirm these findings, Dr. Daryl Carter provided us with additional 24 cases of axillary lymph node metastases from breast carcinoma. Tumor tissue was microdissected, and genomic DNA extracted as described above. Exon 7 of the TßR-I gene was analyzed by PCR-SSCP. In one single case, we detected and confirmed the presence of the identical S387Y mutation found in the initial series. Thus, these results further support our hypothesis that mutations of the TßR-I gene represent relatively late events in breast cancer progression.

Detection of TßR-I and -II Gene Losses by FISH: For cells to loose all responsiveness to TGFß, both alleles of any one of the signaling intermediate genes need to be inactivated. In analogy with other tumor suppressor genes, this is likely to be a two-step process involving loss of one allele and inactivation of the second allele by intragenic mutation. Allelic deletions are often identified by using PCR-based assays for the detection of polymorphic DNA sequences. This approach has several drawbacks: First, it requires the availability of paired tumor- and germline DNA samples. Secondly, such assays are informative only if the individuals are heterozygous for the marker used. Finally, and most importantly, the test will only yield a positive result if the majority of tumor cells has undergone loss of heterozygosity (LOH). Thus, PCR-based approaches will fail to detect allelic losses if they are present in only a minority of the tumor cells.

Fluorescent *in situ* hybridization (FISH) is a particularly attractive alternative method for detecting LOH because it does not require access to normal tissue from the same individual and can be used to detect changes in gene copy numbers in individual cells. Moreover, FISH has been used effectively to detect allelic losses in interphase nuclei in tissue sections or touch preparations of tumor samples.

The main purpose of this study was to determine whether the genes that encode the two TGFß receptors (TßR-I and TßR-II) undergo allelic deletions during breast cancer development and progression. We approached this question by examining interphase nuclei in breast cancer specimens by FISH. A total of 18 primary cancer specimens were examined. These included 15 invasive ductal cancers, 2 invasive lobular carcinomas, and 1 intracystic papillary cancer. Interphase nuclei were hybridized with BAC clones containing the complete genomic sequences of either TßR-I or TßR-II. Specimens were co-hybridized with centromeric probes for the corresponding chromosomes (chromosome 9 for TßR-I, chromosome 3 for TßR-II).

The results for all 18 cases of primary breast cancer have been depicted graphically in **Figure 1**. In most cases, we could identify subpopulations of nuclei in which the number of TßR-specific signals was less than 2. However, the hybridization efficiency of locus-specific DNA probes is probably lower than that obtained with the repeat-sequence probes used to identify centromeres, because the signals are smaller and less intense than centromeric signals. In order to estimate the proportion of false-negative TßR gene signals, we examined touch preparations of 4 different normal axillary lymph nodes that had been obtained at the time of breast surgery and were processed in a manner identical to the tumor samples. The average fraction of nodal lymphocytes with <2 TßR-specific signals was 19% (95% CI: 9-29) for TßR-I and 21% (95% CI: 3-38) for TßR-II. Using the upper boundaries of the 95% confidence intervals as threshold values (29% for TßR-I and 38% for TßR-II), we concluded that tumor cell subpopulations with bona fide TßR-I deletions were present in 2 of 6 (33%), and TßR-II deletions in 6 of 10 (60%) touch preparations . In all cases, approximately half of the losses involved both copies of the TßR-I or -II gene.

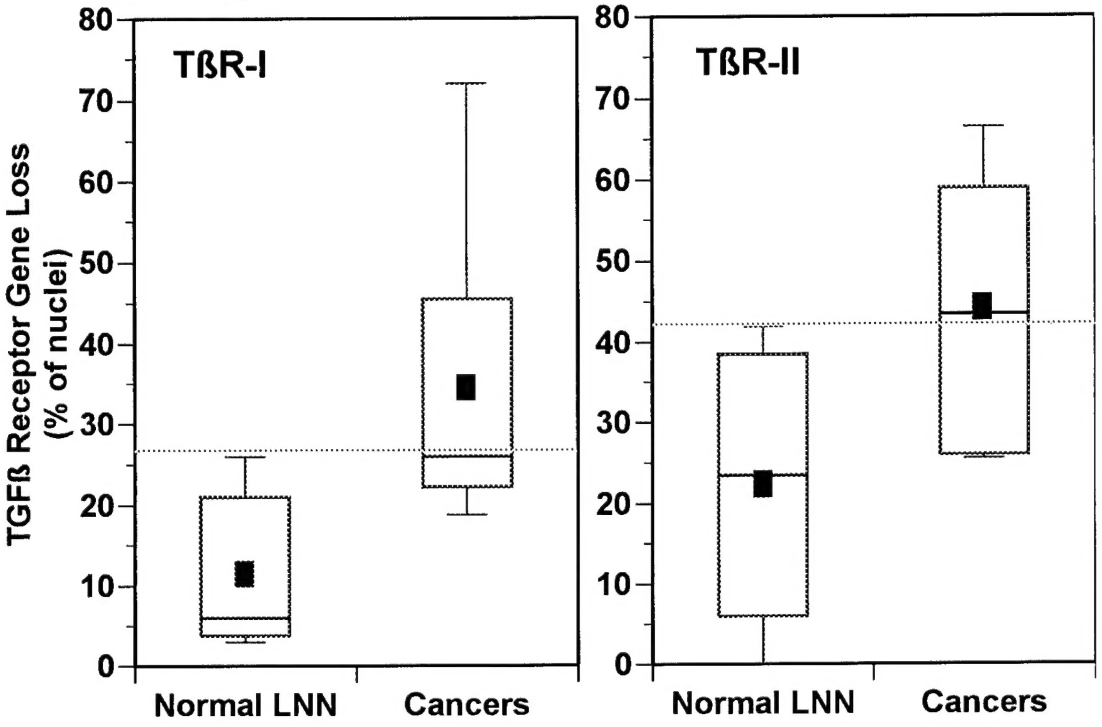

Figure 1. TGFß Receptor Gene Losses in Primary Breast Carcinomas

Our results indicate that approximately 50% of the primary invasive carcinoma specimens contained subpopulations of cells that had undergone allelic losses of either the TßR-I or the TßR-II gene. These findings raised the question at which stage of tumor development these losses had occurred. To answer this question, we will have to examine a series of cases that span the spectrum of pre-invasive to metastatic breast cancer. However, our data provide some preliminary insight. For example, in the single case of non-invasive intracystic papillary carcinoma (6T), we found no evidence of significant allelic loss of either of the two receptor genes. We also examined a single metastatic lesion. This chest wall recurrence demonstrated extensive aneuploidy of both chromosomes 3 and 9 with increasing allelic loss of the TBR genes with increasing chromosome copy number. However, in spite of the high frequency of chromosomal gains and losses, losses of both TßR genes in this case were of the same order of magnitude as those seen in the primary tumors. Thus, these findings suggest that losses of genes that encode TGFß signaling intermediates may occur progressively as breast cancers evolve from pre-invasive to invasive to metastatic lesion.

b. IMMUNOSHISTOCHEMICAL STUDIES:

Generation of anti-phospho-Smad2 antibody: FISH and PCR-SSCP are labor-intensive and technically challenging approaches to identifying lesions in the TGFß signaling pathway that do not lend themselves well to the analysis of large numbers of tumor specimens. Two key features of TGFß signaling can be exploited to gain a better understanding of TGFß signaling in tumor sections. These include the phosphorylation of Smad2 and –3 by activated TGFß receptors, and the nuclear localization of these phosphorylated Smads in transcription complexes. In order to test the validity of this approach, we have developed activation state-specific antibodies directed against

Smad2 and -3. We postulate that loss of expression of Smad4 and/or phosphorylation of Smad2 and -3 accurately predicts the underlying molecular mechanism of TGFß-resistance.

Activation-state specific anti-Smad antibodies: In order to be able to distinguish between the activated (phosphorylated) and inactive forms of Smad2 and -3, we raised polyclonal rabbit antibodies against synthetic peptides comprising the C-terminal 13 amino acids of Smad2 or Smad3, in which two phosphoserine residues were incorporated at the extreme C-terminus (KMGSPSVRCSS^{P}MS^{P} and KMGSPSIRCSS^{P}VS^{P}, respectively), coupled to keyhole limpet hemocyanin (KLH) as carrier protein. The antisera were affinity-purified by negative selection using a KLH-agarose column, followed by chromatography using an Affigel-10 (BioRad) matrix to which unphosphorylated Smad2 and Smad3 peptides had been coupled. The final purification step consisted of a positive selection using the appropriate phosporylated Smad2 (Smad2P) or Smad3 (Smad3P) peptide coupled to Affigel-10 matrix. The antibody was eluted using 3M sodium thiocyanate, immediately neutralized using 100 mM Tris and dialyzed against phosphate buffered saline (PBS) for 48 hours. The specificity and sensitivity of the anti-Smad2P and anti-Smad3P antibodies were confirmed by ELISAs against .BSA conjugates of phosphorylated versus unphosphorylated peptide.

Detection of Smad expression by Western blotting: The sensitivity and specificity of the Smad and phospho-Smad antibodies were tested by Western blotting of extracts of breast carcinoma cells treated with TGFß or vehicle only. Cells were grown to confluence in 100-mm dishes and treated with 100 pM TGFß for 1 hour. Cells were then lysed *in situ* in buffer composed of 150 mM NaCl, 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 1 mM EGTA, 1% (v/v) TritonX-100, 1 mM phenylmethyl sulfonyl fluoride (PMSF, Sigma Chemical Co., St. Louis, MO), 20 µg/mL of aprotinin (Sigma), and 25 µg/mL of leupeptin for 30 min at 4°C. After clarification of the lysates by centrifugation, protein extracts were resolved by SDS-PAGE and transferred to nitrocellulose paper in a buffer composed of 25 mM Tris-HCl (pH 8.0), 192 mM glycine, 20% (v/v) methanol, using an Owl Scientific electroblotting apparatus (USA Scientific Plastics, Ocala, FL). Duplicate filters were then incubated for 30 min. at 20°C in blocking buffer containing PBS supplemented with 5% (w/v) Carnation dry milk and 0.1% (v/v) Tween-20, followed by incubation for 12 to 16 hr at 4°C in PBS containing 1 µg/mL of anti-Smad peptide antibody (Santa Cruz Biotechnology, Santa Cruz, CA) or 1 µg/mL of anti-Smad antibody that had been pre-incubated with a 10-fold molar excess of the cognate peptide. Smad2 and –3 were detected using a goat polyclonal dual specificity anti-Smad2/3 antibody (N-19, Santa Cruz). Smad4 was detected using a mouse monoclonal anti-Smad4 antibody (B-8, Santa Cruz). Smad2P and Smad3P were detected using our own rabbit anti-Smad2P and –3P antibodies (see above). Blots were developed using a 1:2000 dilution of horseradish peroxidase-tagged goat anti-rabbit or –mouse IgG (Calbiochem, San Diego, CA) and the bands visualized using DuPont NEN Chemiluminescence Reagent as recommended by the manufacturer.

We analyzed Smad4 and Smad2P expression in a panel of human breast carcinoma cell lines as well as the non-neoplastic mouse mammary epithelial line, HC-11. Smad4 protein was expressed in HC-11 cells, as well as in 8 of the 10 BC lines. However, 2 of the BC lines (ZR75-1 and MDA-MB-468) failed to express any Smad4 protein. Moreover, TGFß treatment had no effect on Smad4 expression. In contrast, each of the BC lines expressed equal levels of Smad2 (not shown). We then examined the effects of TGFß treatment on Smad2 phosphorylation. While phospho-Smad2 was not detectable in the absence of TGFß, it became easily detectable within 1 h of the addition of 100 pM TGFß1 to HC-11 cells. Phosphorylation of Smad2 was induced by as little as 10 pM of TGFß1, and detectable within 15 min. (data not shown). Besides in HC-11 cells, TGFß treatment induced phosphorylation of Smad2 in 9 of 10 BC cell lines, indicating that these cell lines expressed functionally intact TGFß receptors. In contrast, no phosphorylation of Smad2 was observed in T47D cells, which are known to lack TßR-II expression. Thus, the absence of phospho-Smad2 can be used as a surrogate marker of a TGFß receptor defect.

Tissue microarray studies: In order to assess the status of TGFß signaling in a large cohort of primary human breast carcinoma specimens, we analyzed tissue microarrays that contained a total of 135 cases of primary breast carcinomas. Consecutive sections of the microarrays were stained with hematoxylin-eosin, anti-Smad2 (Santa Cruz), anti-phospho-Smad2, and anti-Smad4 (Santa Cruz). The results are summarized in **Table 1**.

We encountered three types of cases: The majority (88%) expressed both Smad2 and Smad4, as well as activated Smad2P. These findings indicate that biologically active TGFß was present in these tumors, and that the TGFß receptors were actively signaling. In 10 cases (14% of total), Smad2 was expressed, but Smad2P was not detectable. These findings indicate the presence of defective receptors in these cases; we are currently examining these tumors for structural alterations in the TßR-I or –II genes. In addition, 6 of the cases failed to express Smad4 protein. Interestingly, 2 cases that failed to express Smad2P also failed to express Smad4. Recent studies

by Hruban et al have shown that failure to detect Smad4 by immunohistochemistry strongly predicts for the presence of mutation or deletion of the Smad4 gene. Thus, these 2 breast carcinomas appear to have a dual defect in the TGFß pathway that involves both loss of Smad4 and a TßR defect. Similar dual defects have recently been described in several carcinoma cell lines derived from pancreatic-, colorectal-, and head-&-neck cancers.

Table 1. Expression of Smads in human primary breast cancers

	Positive	Negative	Total
Smad2	116 (100%)	0	116
Smad2P	106 (86%)	10 (14%)	116
Smad4	110 (95%)	6 (5%)	116

To investigate the association of expression pattern of Smad2P and Smad4 with other clinical and pathological features of the breast carcinomas, Smad expression patterns were compared with tumor histological grade, nuclear grade, histological type, estrogen- and progesterone receptor and HER2/neu gene expression, as well as lymph node status (**Table 2**). The results failed to demonstrate a significant association between any of these parameters and Smad2P or Smad4 expression.

Table 2. Association between Smad status and breast carcinoma characteristics

	Smad2P pos	Smad2P neg	*P value*	Smad4 pos	Smad4 neg	*P value*
Histological type (n=116)	106	10		110	6	
Ductal	86	9		92	6	
Lobular	20	1	*0.69*	18	0	*0.59*
Histological grade (n=116	107	9		110	6	
I/II	42	4		45	2	
III	65	5	*0.74*	65	4	*1.00*
Nuclear grade (n=116)	107	9		110	6	
I/II	69	6		73	3	
III	38	3	*1.00*	37	3	*0.41*
ER (n=114)	104	10		108	6	
Negative	35	4		36	2	
Positive	69	6	*0.73*	72	4	*1.00*
PgR (n=111)	102	9		105	6	
Negative	37	2		40	3	
Positive	65	7	*0.49*	65	3	*0.66*
HER2/neu (n=102)	94	8		96	6	
Negative	88	7		89	6	
Positive	6	1	*0.45*	7	0	*1.00*
LNN (n=88)	79	9		83	5	
Negative	34	4		30	3	
Positive	45	5	*1.00*	53	2	*0.36*

ER: Estrogen receptor; PgR: Progesterone receptor; LNN: Lymph nodes

In order to investigate the possibility that hereditary breast cancers may demonstrate a higher frequency of TGFß receptor inactivation than sporadic cases, we analyzed a panel of primary breast cancers obtained from women <35 of age whose germlineBRCA1 and -2 gene status had been determined by direct sequencing. As summarized in Table 3, the frequency of Smad2P-negative cases (indicative of TßR defect) was similar among cases bearing germline BRCA gene mutations and age-matched BRCA wild type controls. Thus, in contrast to hereditary non-polyposis colorectal cancers, in which the frequency of TßR-II mutations is 80%, BRCA gene mutations do not appear to predispose to TßR gene inactivation.

Table 3. Smad expression and activation in hereditary breast carcinomas

BRCA status	Smad2P Positive	Smad2P Negative	Total
Wild type	33 (89%)	4 (11%)	37
Mutant	13 (93%)	1 (7%)	14
Total	46	5	51

Smad2P expression in human breast carcinoma specimens as a function of germline BRCA genotype. Numbers in parentheses indicate percent of total. Two-tailed p-value: 1.0000 (Fisher's Exact test).

Task 2. Determination of the functional consequences of TGFß-receptor mutations

In order to test whether the serine to tyrosine substitution at position 387 found in primary and metastatic breast cancer specimens disrupts receptor function, we introduced this mutation into a full-length wild type TßR-I cDNA. We studied the effects of the mutation on receptor function in transient transfection assays using the TßR-I-deficient R-1B (L17) mink lung epithelial cell line. Expression of wild type TßR-I in R-1B (L17) cells resulted in an approximately 50% reduction in cyclin A promoter activity compared to cells transfected with an inert control vector. In contrast, pCAL2 activity was repressed by less than 30% in cells transfected with the S387Y receptor mutant. In cells transfected with wild type TßR-I, pSBE4-dependent luciferase activity was increased approximately 15-fold over controls, while the increase observed in cells transfected with the receptor mutant was only approximately 10-fold. The S387Y mutation appears to induce a shift in the TGFß dose-response relationship: wild type TßR-I expressing cells responded maximally to 50 pM TGFß, whereas S387Y expressing cells required at least 100 pM TGFß for maximal response. Repression of the cyclin A promoter activity (pCAL2) correlates extremely well with the ability of cells to respond to TGFß-mediated cell cycle arrest, and activation of the Smad DNA-binding element (SBE) in pSBE4 reflects TGFß-induced gene transcription. To rule out that the observed differences in reporter gene activity were due to variations in levels of expression of wild type and mutant TßR-Is in transfected cells, cell lysates were subjected to Western immunoblotting using anti-HA monoclonal antibody. Discreet 55 kDa bands of equal intensity corresponding to the TßR-I receptor were detected in extracts from both wild type- and mutant TßR-I-transfected cells. Thus, the S387Y mutation did not affect receptor protein expression. In summary, cells expressing the S387Y mutant was significantly less sensitive to the effects of TGFß on cell cycle regulation as well as transcriptional responses than cells expressing the wild type receptor.

The exact mechanism whereby the S387Y mutation diminishes TGFß signaling remains to be determined. According to the canonical domain subdivisions found in all protein kinases, the serine residue at position 387 in TßR-I is located in the linker region between subdomains VIII and IX which typically form the peptide recognition domain of protein kinases. The structure of subdomains VIII and IX are highly conserved among the family of type I TGFß-, activin- and bone morphogenic protein (BMP) receptor serine-threonine kinases. The fact that these receptors share highly homologous substrates (Smads) further suggests that this region participates in substrate recognition. Alternatively, it may well affect the homodimerization of TßR-I molecules, or perhaps the interactions between TßR-I and –II molecules when they form heterotetrameric complexes during receptor activation.

The primary substrate of TßR-I, Smad2, is phosphorylated on two serine residues located within the consensus sequence RCSS(465)MS(467) at the C-terminus of the protein. Although the C-terminal tail of Smad2 is not absolutely required for its physical interaction with TßR-I, structure-function studies indicate that it clearly plays a complimentary role in enzyme-substrate recognition and partly determines specificity between TGFß and

BMP signaling. Comparison between the crystal structures of activated protein kinase A in complex with an inhibitory peptide and that of the TßR-I kinase indicates that the S387Y residue does not fall precisely within the canonical substrate binding site as defined in the protein kinase A-inhibitory peptide structure. However, this does not exclude the possibility that the substitution of a tyrosine with its larger side chain for the serine at position 387 in TßR-I interferes with productive substrate recognition, particularly as the interface between a Smad and TßR-I is probably much larger than the protein kinase A-inhibitory peptide interface. Furthermore, based on the Chou and Fassman algorithm, one would predict that the S387Y mutation alters the secondary structure of the TßR-I kinase by introducing two ß-sheets flanking the loop that connects the E and F α-helices of the catalytic core. It is worth noting that two other TGFß type I receptors, TSR-1 and TskL7, contain different polar residues at position 387 (threonine and glutamine, respectively). Interestingly, neither of these two receptors is able to elicit the same cellular responses as TßR-I, perhaps because they are unable to interact with Smad2 or –3.

Finally, the functional importance of this region is also illustrated by the fact that several syndromes have been associated with mutations within subdomains VIII or IX in other protein kinases. For example, two different arginine-to-tryptophan and methionine-to-arginine mutations in the TSR-1 gene have been described in hereditary haemorrhagic telangiectasia type 2. Moreover, amino acid substitutions at highly conserved glutamate and aspartate residues in the catalytic subunit of phosphorylase B kinase result in loss of enzyme activity, glycogenosis and liver cirrhosis. In addition, Wang et al. recently described a case of head-&-neck cancer with a tyrosine-to-cysteine mutation within subdomain IX of the TßR-II serine-threonine kinase. Although the effects of this mutation on receptor function were not reported in this case, it is likely that it affects enzyme activity as well.

In summary, we have identified a single missense mutation of the TßR-I gene that occurs with relatively high frequency in invasive ductal breast cancer and that has a significant negative impact on receptor signaling. This is the first reported missense mutation in this gene reported in any human malignant neoplasm and provides further support for the idea that inactivation of the TGFß signaling pathway can play an important role in human carcinogenesis. Furthermore, the high frequency of the S387Y mutation in lymph node metastases suggests that inactivation of this signaling pathway may be particularly associated with the metastatic phenotype.

Task 3. To determine the potential clinical significance of genetic alterations of the TßR genes in breast cancer

We have addressed the clinical significance of TGFß signaling in two different ways:

First, we are in the process of analyzing the effect of TGFß receptor defects and Smad4 inactivation and long-term outcome in our tissue microarray study (see above). These results will be included in the manuscript that is being prepared.

Secondly, we had proposed to test the hypothesis that the actions of the anti-estrogen, tamoxifen, are mediated by the induction of biologically active TGFß. If this assumption is correct, we predicted that defects in TGFß signaling might explain the cases of tamoxifen resistance among estrogen receptor-positive breast cancers. We have begun to address these questions in a preliminary study conducted in collaboration with Drs. Lorna Marson and William Miller of the University of Edinburgh. These investigators have compiled a series of breast biopsies taken from patients prior to and 6 months following the start of tamoxifen therapy. We have examined the TGFß signaling pathway using our immunohistochemical approach in 10 paired specimen sets obtained from Edinburgh. The results are summarized in **Table 4**.

The results of this pilot study indicate that each of the 9 evaluable cases expressed Smad2P. However, in the 4 non-responders, Smad2P immunostaining was not increased in the post-treatment samples, whereas Smad2P expression was clearly increased in 2 of the 4 evaluable responders. Although this small feasibility does not allow us to draw any conclusions, we intend to complete an analysis of the entire cohort of 100 paired specimens from Edinburgh.

Table 4. Smad2 activation in breast cancers as a function of tamoxifen therapy

#	Histology	Smad2P expression	Response?
1A	No cancer No normal ducts	N/A N/A	
1B	Normal ducts DCIS	1-2+ 1-2+	Yes
2A	Invasive carcinoma	1+	
2B	Normal ducts DCIS Invasive carcinoma	0-1+ 0-1+ 1+	No
3A	Invasive carcinoma (mucinous)	1+	
3B	Skin Normal ducts Invasive carcinoma	2+ 1-2+ 1-2+	Yes
4A	DCIS Invasive carcinoma	1-2+ 1-2+	
4B	Normal ducts ADH Tubular carcinoma	0-1+ 0-1+ 0	Yes
5A	Normal ducts Invasive carcinoma	1+ 1-2+	
5B	Normal ducts No carcinoma seen	0	Yes
6A	Tubular carcinoma	1+	
6B	Tubular carcinoma	1-2+	Yes
7A	Normal ducts DCIS Invasive carcinoma	0 0-1+ 1-2+	
7B	Normal ducts ADH DCIS Invasive carcinoma	0 0 0 1+	No
8A	Normal ducts Invasive carcinoma	1-2+ 1-2+	
8B	Normal ducts Invasive carcinoma	0 0	No
9A	Normal ducts Invasive carcinoma	1-2+ 1-2+	
9B	Invasive carcinoma	0	No

Key research accomplishments:

- Detection of allelic losses of TGFß receptor genes in primary human breast cancer
- Identification of the first missense mutations in the TGFß type receptor gene in human (breast) cancer
- Identification of TßR receptor defects and Smad4 losses in primary human breast cancer tissue microarrays

Reportable outcomes:

- Generation of phospho-Smad2-specific antibody
- **Reiss, M.** and Barcellos-Hoff, M.H. Transforming Growth Factor-ß in breast cancer-a working hypothesis-Breast Cancer Res. & Treatment. 1997. 45:81-95.
- Chen, T., Carter, D., Garrigue-Antar, L., and **Reiss, M.** Transforming Growth Factor-ß type I receptor kinase mutant associated with metastatic breast cancer. Cancer Res. 1998. 58:4805-4810.
- Rimm, D.L., Camp, R.L., Charette, L.A., Costa, J., Olson, D., and **Reiss, M**. Tissue microarrays: A new technology for amplification of tissue resources. Cancer J. 2001, 7:24-31.
- Xie, W., Mertens, J.C., Reiss, D.J., Rimm, D.L., Camp, R., Haffty, B., and **Reiss, M**. Alterations of Smad signaling in sporadic and hereditary human breast cancer-A tissue microarray analysis. In preparation

CONCLUSIONS

Based on the observation that most cell lines derived from human breast carcinomas are refractory to TGFß-mediated cell cycle arrest *in vitro*, we wished to address the hypothesis whether human breast carcinomas *in vivo* have also escaped from TGFß-dependent cell cycle control. If the hypothesis was correct, we intended to determine the underlying molecular mechanisms, the stage of tumor development at which escape from TGFß control typically occurs, and the possible clinical implications of our findings.

We have made substantial progress in answering each of these questions. Perhaps the most important observation is that the vast majority of primary human breast cancers express the activated (phosphorylated) form of the TGFß signaling molecule, Smad2. The presence of Smad2P is a highly specific and sensitive indicator of a cellular response to bioactive TGFß; thus, the presence of Smad2P in breast carcinomas proves that biologically active TGFß is binding to and activating cell surface receptors on the cells. As these tumor cells are clearly actively proliferating, our observations represent the first clear indication that human breast cancers in vivo are no longer subject to TGFß-mediated cell cycle arrest. Furthermore, this observation indicates that, by the time the primary invasive breast cancer has developed, it is already composed of TGFß-resistant cells. Future studies will address the question at which (pre-invasive) stage of development of breast cancer this escape from TGFß occurs.

Our second major finding is that inactivation of TGFß receptors and/or loss of Smad4 occurs in only approximately 10% of primary breast cancers. Thus, in the majority of cases, mechanisms other than receptor and/or Smad inactivation appear to be responsible for the escape from TGFß-dependent cell cycle control. In future studies, we plan to focus on the portion of the TGFß signaling pathway that is downstream of Smad4, particularly on the genes involved in TGFß-mediated transcriptional regulation of cell cycle genes.